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The impact of imiquimod on radiation-induced lung injury: Results of an experimental study

Imiquimod attenuates radiation lung injury

Mustafa Calik[1], Güler Yavaş[2], Zeliha Esin Çelık[3], Çağdaş Yavaş[2], Saniye Goknil Calık[4], Mustafa Fevzi Sargon[5], Hidir Esme[1]
[1]Department of Thoracic Surgery, Health Sciences University, Konya Training and Research Hospital, Konya,
[2]Department of Radiation Oncology, Selcuk University Faculty of Medicine, Konya,
[3]Department of Pathology, Selcuk University Faculty of Medicine, Konya,
[4]Department of Emergency and First Aid, KTO Karatay University Vocational School of Health, Konya,
[5]Department of Anatomy, Hacettepe University Faculty of Medicine, Ankara, Turkey

Abstract

Aim: Radiation-induced lung injury (RILI) is a major dose-limiting factor during thoracic irradiation. Imiquimod (Imq) is a heterocyclic amine that has been shown to be effective in diseases related to inflammation and fibrosis. In this study, we aimed to evaluate the impact of Imiquimod on RILI. Material and Method: The study included 60 adult female Wistar-Albino rats (250-300 g). Rats were divided into 6 groups: Group (G) 1: control, G2: radiotherapy (RT) only, G3 and G4: 5 and 10 mg/kg Imq; G5 and G6 RT: plus 5 and 10 mg/kg Imq groups respectively. A single dose of 15 Gray (Gy) RT was given to the lungs. Imq was applied intraperitoneal. Results: The inflammation, fibrosis, and transforming growth factor (TGF)-β scores of the study groups were significantly different at 6th and 16th week of RT ($p< 0.001$ for all). At the 6th week of RT, inflammation, fibrosis, and TGF- β scores did not differ in both RT and non-RT groups. By the 16th week of RT inflammation, fibrosis, and TGF- β scores were significantly different between G2 and G5, and G2 and G6. Electron microscopy findings supported the results of the light microscopy. Discussion: Although Imq did not improve pneumonitis phase, Imq attenuated radiation-induced lung fibrosis. These findings should be clarified with further preclinical and clinical studies.

Keywords

Imiquimod; Fibrosis; Lung; Pneumonia; Radiotherapy

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Corresponding Author: Mustafa Calik, Department of Thoracic Surgery, Health Sciences University, Konya Training and Research Hospital, 42090, Meram Konya, Turkey. T.: +90 3323236709 F.: +90 3323236723 E-Mail: drmcalik@hotmail.com
ORCID ID: https://orcid.org/ 0000-0001-9963-5724

Introduction

Radiation-induced lung injury (RILI) is a major dose- limiting toxicity for patients receiving radiation to the thorax [1]. The mechanism of RILI is not completely understood; however, there are numerous cells, mediators and signaling pathways that are involved in the initiation and progression of RILI, suggesting multiple potential complex mechanisms for the prevention and treatment of this disease. Radiotherapy (RT) causes an oxidative stress and free radical production that leads to an inflammatory response and subsequent DNA damage. The resulting injury or apoptosis of alveolar epithelial cells and vascular endothelial cells then induce a series of inflammatory reactions and chemotaxis of monocytes, lymphocytes, and granulocytes, which gather at the site of tissue injury. Subsequently, high levels of circulating platelet-derived and basic fibroblast growth factors can be predominant and lead to fibroblast proliferation and migration, transforming growth factor-β (TGF-β) upregulation and collagen deposition, which eventually causes to ventilation-perfusion mismatches and late declines in pulmonary function [2-4]. It is thought that various cytokines and related factors have been implicated in the pathogenesis of radiation fibrosis, including interleukin-1 (IL-1), IL-4, IL-6, IL-13, Interferon-gamma (IFNγ), and tumor necrosis factor-α /TNF-α). TGF-β1 plays an integral role in fibrosis formation by promoting the chemo-attraction of fibroblasts and their conversion to myofibroblasts [2,4,5].

Clinically RILI is divided into two phases: (1) pneumonitis and (2) fibrosis. The radiation pneumonitis is rarely encountered during the treatment since it usually develops 1-6 months following RT (2). Radiation pneumonitis is usually manifested with low-grade fever, dyspnea and, non-productive cough. Progressive radiation pneumonitis may cause chronic pulmonary damage or acute respiratory distress that can be life-threatening. The differential diagnosis of radiation pneumonitis and, radiation fibrosis is often not possible, since these two entities may constitute a continuous spectrum of RILI. Moreover, the development of radiation pneumonitis may increase the risk for the development of radiation fibrosis [2, 3, 6].

Imiquimod (Imq) is a non-nucleoside, low molecular weight, heterocyclic amine (imidazoquinolone) with the chemical structure 1-(2 methylpropyl)-IH-imidazo [4,5-c] quinolin-4-amine [7]. Imq was first shown to be a potent inducer of cytokines using the guinea pig model of herpes simplex virus (HSV) infection [8]. The exact mechanism of action in human beings is still unknown; however, in general application, Imq stimulates multiple proinflammatory cytokines including IFN-α, IFN- γ and (tumor necrosing factor) TNF-α, which increases collagen breakdown and reduces fibroblast-mediated collagen production via reducing TGF-β [9]. The stimulation of pro-inflammatory cytokines by Imq induces a profound tumor-directed cellular immune response [7, 10]. The molecular basis of this pro-inflammatory activity of imiquimod is probably related to a toll-like receptor (TLR)-directed agonistic activity [10, 11]. It has been demonstrated that Imq has a beneficial effect in the prevention of postsurgical keloid recurrence, [12, 13, 14] solar keratosis, cutaneous tumors including mycosis fungicides, superficial basal cell carcinoma, [15] human papillomavirus (HPV)-induced genital warts, [16] non-alcoholic fatty liver disease-related inflammation, and fibrosis by TLR-directed pathway, [17] induces expression of various inflammatory genes in the hypothalamus [18]. The present study was designed to determine whether imiquimod treatment would attenuate RILI.

Material and Method

Study Design

The study included 60 adult female Wistar-Albino rats (250-300 g). All institutional and national guidelines for the care and use of laboratory animals were followed. In addition, all animals received humane care and were used in compliance with standards established by the European Convention for Animal Care and Use of Laboratory Animals. The rats were fed with a standard pelleted diet and were allowed to access tap water ad libitum. The animals were housed in 4 per cage cages on a 12 -hour h light/dark cycle at room temperature in a humidity-controlled environment. The 60 female Wistar rats included in the study were randomly grouped into six groups containing 10 rats each. Group (G) 1 was defined as a control group in which the rats were sham irradiated. G2 was the RT only group receiving a single dose of RT (15 Gy), but no medication. G3 and G4 were 5 mg/kg and 10 mg/kg Imq groups and rats in these groups were given Imq (R837; InvivoGen, San Diego, CA, USA) in a single dose of 5 mg/kg and 10 mg/kg daily intraperitoneal injection for 16 weeks respectively. G5 and G6 were defined as 5 mg/kg Imq + RT, and 10 mg/kg Imq + RT groups and rats in these groups were given Imq in a single dose of 5 mg/kg and 10 mg/kg daily intraperitoneal injection for 16 weeks, starting 48 hours before the administration of 15 Gy single dose RT (Table 1). The rats from G1 and G2 were given NaCl intraperitoneally in the same timing procedure with Imq groups (G3, G4, G5, and G6). The local Animal Ethical Committee of this study has approved all study-related procedures. This study was approved and funded by The School of Medicine Animal Care and Investigational Committee at our institution which we gratefully acknowledge. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Table 1. The abbreviations used for the study groups

Group (G)	
G1	Sham-irradiated control group
G2	15 Gy group
G3	5 mg/kg Imiquimod group
G4	10 mg/kg Imiquimod group
G5	15 Gy +5 mg/kg Imiquimod group
G6	15 Gy + 10 mg/kg Imiquimod group

Irradiation protocol

RT was applied under general anesthesia with intraperitoneally administered 90 mg/kg ketamine hydrochloride (Ketanest, Pfizer Pharma GmbH, Karlsruhe, Germany) and 10 mg/kg xyalazine (Alfazyne 2%; Alfasan International. BV, Woerden, Netherlands). A single dose of 15 Gy with 6 MV photon beams was applied via a single anterior field to 2 cm depth with SAD (source-axis distance) technique. Elasto-gel bolus (1 cm) was used to build up the radiation dose on the lungs and to provide contour regularity. The field size was 4x4 cm and included both lungs.

Imiquimod protocol

Lyophilized Imiquimod (R837; InvivoGen, San Diego, CA, USA) was reconstituted in sterile endotoxin-free water at a concentration of 1 mg/ml according to the manufacturer's instructions. Doses of 5 mg/kg or 10 mg/kg were used for intraperitoneal injections [18]. Imq was started 48 hours before the irradiation and continued daily doses until the animals were sacrificed.

Morphologic Studies

At the 6th and 16th weeks of the RT 5 animals from each group were anesthetized and sacrificed by cervical dislocation, and both lungs were removed. The lung tissues were examined with both light and electron microscopies.

Histopathologic evaluation

The bilateral whole lungs of each rat were excised and fixed in 10% neutral buffered formalin. Two lobes of each lung for each rat were processed and embedded in paraffin for light microscopic examination. The 4 µ thick sections obtained with microtome were stained with haematoxylin and eosin (H&E) to evaluate the inflammation, and with histochemical Masson Trichrome staining to identify the fibrosis in the lung. Extent of the chronic inflammatory cells including lymphocytes on alveolar walls was graded on a scale from 0 (normal) to 3 (severe). Fibrosis was defined as the thickened alveolar walls with superimposed collagen. As a quantitative endpoint, extent of the radiation-induced fibrosis was graded on a scale from 0 (normal lung or minimal fibrous thickening) to 4 (total fibrous obliteration of the field) as described in Table 2 [19]. The pathologist was not aware of the treatment groups at the time of the histological examination of the specimens. After examining the whole sections for each rat, the average value of fibrosis and chronic inflammation per rat was taken as the fibrosis and inflammation scores and mean values of each group were_calculated.

Table 2. The scoring system for lung fibrosis

Grade of fibrosis	Histological features
0	Normal lung or minimal fibrous thickening of alveolar or bronchial walls
1	Moderate thickening of the wall without obvious damage to lung architecture
2	Increased fibrosis with definitive damage to lung structure and formation of fibrous bands or small fibrous masses
3	Severe distortion of the structure and large fibrous areas; "honeycomb lung" is placed in this category
4	Total fibrous obliteration of the field

Ashcroft T, Simpson JM, Timbrelli V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol. 1998;41:467-470 (20).

Immunohistochemistry staining and scoring procedure

Paraffin-embedded tissues of chosen slides were collected and 4 µm thick sections were prepared for immunohistochemistry. The sections were deparaffinized at 37 ºC oven overnight. Immunohistochemical staining was performed using an automatic staining machine (Ventana, Benchmark XT). The sections were boiled at sodium citrate buffer at 95 ºC for 60 min and then incubated with primary antibody Anti TGF-β rabbit polyclonal antibody (ABCAM, ab92486, Cambridge, UK) at a dilution of 1:100 for 52 minutes. The sections were incubated with the secondary antibody for 20 min at room temperature, incubated with Ultra I-view detection kit and counterstained with hematoxylin for 8 min.

The immunohistochemical TGF-β stained slides were evaluated and scored by a single pathologist blinded to patients' data. On the light microscopic evaluation of each 4 µm thick section, 10 different fields magnified 100X were reviewed. Immuno-reactivity scoring system (IRS) which was previously described by Wang et al. [20] was used to determine TGF-β expression levels. This system depends on multiplication of staining intensity and TGF-βpositive alveolar cell percentage. The percentage of positive cells was scored as follows: 0, negative; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100% and the staining intensity as 0 (-), 1 (+), 2 (++), 3 (+++).

Ultrastructural evaluation with electron microscopy

The tissue samples which were taken from the animals 6 and 16 weeks after RT were put into 2.5% glutaraldehyde for 24 hours for primary fixation. The same application was done to the rats of the Imq and control groups after the administration of the drug or NaCl in the same timing procedure. Then, these samples were washed with Sorenson's Phosphate Buffer solution (pH: 7.4) and they were post-fixed in 1% osmium tetroxide. After post-fixation, they were washed with the same buffer and dehydrated in increasing concentrations of alcohol series. After dehydration, the tissues were washed with propylene oxide and embedded in epoxy resin embedding media. The semi-thin and ultrathin sections of the obtained tissue blocks were cut with an ultramicrotome (LKB Nova, Sweden). These semi-thin sections which were 2 micrometers in thickness were stained with methylene blue and examined under a light microscope (Nikon, Japan). Following this procedure, trimming was done to the tissue blocks and their ultrathin sections which were about 60 nanometers in thickness were taken by the same ultramicrotome. These ultra-thin sections were stained with uranyl acetate and lead citrate and they were examined under Jeol JEM 1200 EX (Japan) transmission electron microscope. The electron micrographs of the specimens were taken by the same microscope.

Statistical Method

The Statistical Package for Social Sciences (SPSS) 18.0 portable for Windows (SPSS Inc, Chicago, Illinois, USA) was used for statistical analyses. As the pathological scores were ordinal in nature, the differences in pathological findings between the study groups were analyzed using the Kruskal-Wallis test. When an overall statistically significant difference was observed, pairwise comparisons were performed using the Mann-Whitney U test. Bonferroni correction was used for multiple comparisons. A 5% type-I error level was used for the statistical significance cut-off for overall comparisons. $P<0.05$ was considered for statistical significance level.

Results

Histopathological and immunohistochemical findings at the 6th week of RT

The inflammation, fibrosis, and TGF-β scores between study groups were significantly different at the 6th week of RT ($p< 0.001$ for all) (Table 3). The tissue samples taken from the RT groups had higher scores of all parameters when compared to non-RT groups. Therefore, we performed pair-wise comparisons separately for RT groups and non-RT groups. Among RT groups, the mean inflammation, fibrosis, and TGF-β scores were not significantly different (Table 4). In all RT groups there were moderate inflammation and fibrosis.

In the non-RT groups, there weren't any pathological findings. The histopathological examination of both low-dose Imq group and high-dose Imq group was similar with the control group and there was no statistically significant difference between the non-RT groups (Table 4). The histological sections of the rats' lung exhibited alveolar structures surrounded by the dense capillary nets. Alveoli were separated from each other by thin alveolar septums and covered by the layer of epithelial cells.

Histopathological and immunohistochemical findings at the 16th week of RT

At the 16th week of RT, histopathological and immunohistochemical evaluation revealed that there were significant differences between the study groups in terms of the inflammation, fibrosis, and TGF-β scores ($p< 0.001$ for all) (Table 3). Similar to the 6th-week findings, the RT groups had higher scores of all parameters when compared to non-RT groups. The inflammation, fibrosis and, TGF- β scores of RT only group were significantly higher than low-dose Imq + RT group and, high-dose Imq +RT groups (p-values were, G2-G5: 0.033, 0.041, and <0.001; and G2-G6: 0.043, 0.002 and, <0.001 for inflammation, fibrosis and, TGF-β scores respectively) (Figure 1). There wasn't any difference with respect to all parameters between low-dose Imq +RT and, high-dose Imq + RT groups (Table 4) (Figure 2). There weren't any pathological findings in non-RT groups at the 16th weeks of RT.

Ultrastructural findings at the 6th week of RT

Ultrastructural examination with electron microscopy revealed that there weren't any pathologic changes in non-RT groups at the 6th week of RT. In the non-RT groups, the alveolocapillary membrane, bronchi, and bronchioles were ultrastructurally normal. There was no vacuole in the alveolar epithelial cells.

Table 3. The semiquantitative scoring of chronic inflammation, fibrosis and TGF-β staining at 6th and 16th weeks of RT

Group	*Inflammation (mean±SD)	*P	**Fibrosis (mean±SD)	**P	***TGF-β staining (mean±SD)	***P
6th week of RT						
Control Group	0.1±0.33		0.2±0.44		1.4±0.52	
RT only group	2±0.82		2.1±0.87		5.2±1.98	
Low-dose I	0.2±0,42	<0.001	0.2±0.42	<0.001	1.7±0.48	<0.001
High-dose-I	0.2±0,42		0.2±0,42		2.1±0.57	
RT +Low-dose I	2.1±0.57		2.1±0.32		6.2±0.63	
RT + High-dose I	2.2±0,52		2.2±0.42		6.1±0.74	
16th week of RT						
Control Group	0.2±0.42		0.3±0.48		1.5±0.53	
RT only group	2.8±0.42		3.6±0.52		10.9±0.99	
Control Group	0.4±0.52	<0.001	0.3±0.48	<0.001	1.5±1.052	<0.001
RT only group	0.3±0.48		0.3±0.48		2.4±0.84	
Low-dose I	2.1±0.51		2.8±0.63		7.3±0.82	
High-dose-I	2.0±0.42		2.6±0.69		6.3±0.94	

*: Scores for chronic inflammation are: 0=none, 1=mild, 2=moderate, 3=severe; **: Scores for fibrosis are: 0=none, 1=mild, 2=moderate, 3=severe, 4=total lung fibrosis; ***Scores of TGF-β are: 0 (-), 1 (+), 2 (++), 3 (+++).

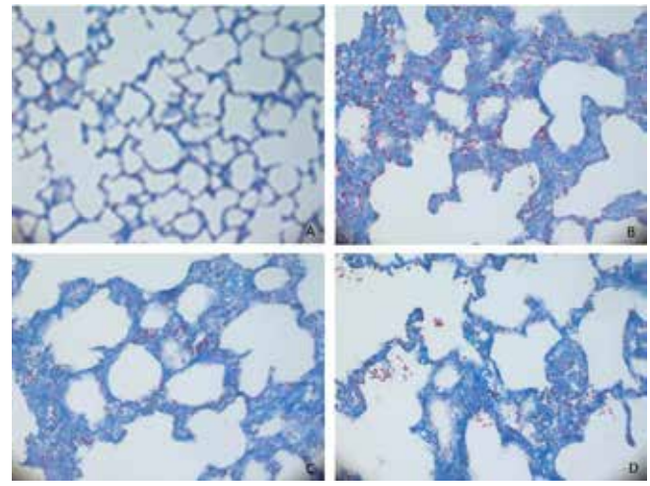

Figure 1. Histochemical Masson Trichrome staining (200X): Section from the lungs of the rats from a) Control group; b) RT only group; c) RT + 5 mg/kg imiquimod; d) RT + 10 mg/kg imiquimod at the 16th week of RT.
a) Control group: Normal alveolar structures with thin alveolar walls.
b) RT only group: There were thickening in the alveolar walls with severe fibrosis and partial lung obliteration.
c) RT + 5 mg/kg imiquimod: There were thickening in the alveolar walls with moderate fibrosis.
d) RT + 10 mg/kg imiquimod: There were thickening in the alveolar walls with moderate fibrosis.

Table 4. Pair-wise comparisons of the RT groups regarding to inflammation, fibrosis, and TGF-β scores at 6th and 16th weeks of RT

Pair-wise Comparison Groups 6th week			p*	
Group 1	Group 2	Inflammation	Fibrosis	TGF-β
Non-RT groups				
G1	G3	0.916	0.994	0.983
G1	G4	0.916	0.994	0.323
G3	G4	0.996	0.999	0.742
RT groups				
G2	G5	0.996	0.890	0.906
G2	G6	0.919	0.994	0.983
G5	G6	0.674	0.994	0.984
Pair-wise Comparison Groups 16th week			p*	
Group 1	Group 2	Inflammation	Fibrosis	TGF-β
Non-RT groups				
G1	G3	0.928	0.999	0.999
G1	G4	0.997	0.999	0.112
G3	G4	0.997	0.999	0.112
RT groups				
G2	G5	0.033	0.041	<0.001
G2	G6	0.043	0.002	<0.001
G5	G6	0.928	0.819	0.119

G=group, RT=radiotherapy, TGF-β=Transforming growth factor-beta
*: Bonferroni Correction was used to evaluate the significance levels of Type-I error for pair- wise comparisons

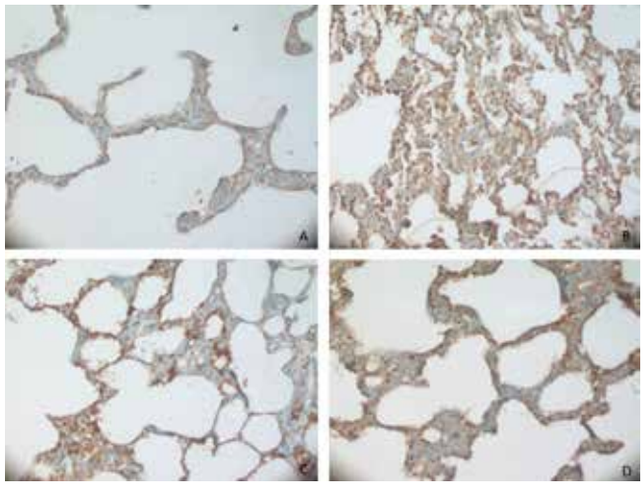

Figure 2. Immunohistochemical TGF-β staining (200X): Section from the lungs of the rats from a) Control group; b) RT only group; c) RT + 5 mg/kg imiquimod; d) RT + 10 mg/kg imiquimod group at 16th week of RT.
a) Control group: There were a few TGF-β positive alveolar epithelial cells or fibroblasts with staining of low intensity.
b) RT only group: There were intense and diffuse TGF-β positivity in alveolar epithelial cells or fibroblasts.
c) RT + 5 mg/kg imiquimod group: TGF-β positivity was lower than group b.
d) RT + 10 mg/kg imiquimod: TGF-β positivity was lower than group b and c.

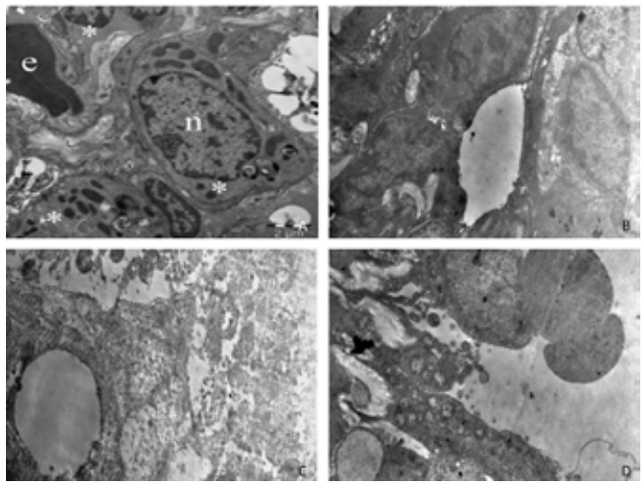

Figure 3. Ultrastructural findings with electron microscopy at the 16th week of RT (a) Control group (b) RT only group (c) RT+5 mg/kg imiquimod group (d) RT+ 10 mg/kg imiquimod group:
(a) Control group: Electron micrograph showing alveolar epithelial cells (*), nucleus of an alveolar epithelial cell (n) and an erythrocyte (e) inside the alveolar capillary in the lung of a control group rat.
(b) RT only group: Many vacuoles were observed in the alveolar epithelial cells and capillary endothelial cells. Intercellular edema was present in between the alveolar epithelial cells.
(c) RT + 5 mg/kg imiquimod group: Many vacuoles were observed in the alveolar epithelial cells and capillary endothelial cells. Intercellular edema was present in between the alveolar epithelial cells. However, the vacuoles and intercellular edematous areas were smaller than RT only group.
(d) RT + 10 mg/kg imiquimod group: Many vacuoles were observed in the alveolar epithelial cells and capillary endothelial cells. Intercellular edema was present in between the alveolar epithelial cells. However, the vacuoles and intercellular edematous areas were smaller than RT only group.

There was no intercellular edema in the bronchioles and lower respiratory systems.

In RT only group, there were many large vacuoles in the alveolar epithelial cells. Additionally, small vacuoles were seen in the cytoplasm of capillary endothelial cells. There was moderate intercellular edema in-between the alveolar epithelial cells. In low-dose Imq +RT and, high-dose Imq +RT groups we observed some vacuoles in the alveolar epithelial cells in addition to capillary endothelial cells. Pericellular edema was present around the alveolar epithelial cells. However, the vacuoles and intercellular edema were smaller than RT only group. There was not any difference between low-dose Imq +RT and, high-dose Imq +RT groups after 6 weeks of RT.

Ultrastructural findings at the 16th week of RT

The sixteenth-week examination of the rats from the non-RT groups demonstrated that there were no ultrastructural pathological findings. The ultrastructural examination of RT only group revealed many vacuoles in the alveolar epithelial cells and capillary endothelial cells. There was severe intercellular edema in between the alveolar epithelial cells. The vacuoles were larger and intercellular edema was more severe than the 6th week findings of the RT only group. At the 16th week of RT, the low-dose Imq +RT group had the same ultrastructural findings with low-dose Imq +RT group at the 6th-week of RT. Similarly, both the 6th and the 16th- week ultrastructural findings of high-dose Imq +RT group were same (Figure 3).

Discussion

Radiation therapy is an essential treatment modality for breast cancer, lung cancer, esophageal cancer, and other thoracic cancers. However, the efficacy of RT can be severely compromised by the side effects of RILI including pneumonitis and lung fibrosis, which eventually deteriorate the quality of life of the patients. Modern treatment techniques, including intensity modulated RT (IMRT), image-guided RT (IGRT) and stereotactic body RT (SBRT) are still limited by the toxicity of nearby normal tissues, thus impending further gains with these technologies. Therefore, RILI is of critical importance and major dose- limiting factor for a radiation oncologist in attempting to deliver curative doses to the tumor. Imq, the first of a new class of compounds called “immune system modifiers” stimulates the innate and adaptive immune pathways and induces cytokine production. It is used for many conditions including genital and perianal warts, postsurgical keloid recurrence, solar keratosis, and cutaneous, non-alcoholic fatty liver disease. Imq induces the cytokines IFN-α and IFN- γ, TNF-α, IL-1, 5, 6, 8, 10 and 12. However, it is the induction of IFN-α and - γ, that makes Imq particularly suited for the anti-fibrotic purpose [21]. It was demonstrated Imq inhibits human fibroblast collagen production by IFN-α and – γ in a dose-dependent manner [22]. IFN-α and IFN-γ reduce both collagen and fibrosis. Moreover, IFN- γ may down-regulate the profibrotic Th2 cytokine IL-4, in addition to down-regulating the expression of TGF-β, which is the master chief of the radiation-induced fibrosis [2, 21, 23]. By the light of these findings, we hypothesized that Imq may have a protective effect on RILI. To the best of our knowledge, the current study is the first one investigating the role of Imq in RILI. We demonstrated that Imq attenuated “radiation-induced lung fibrosis” without ameliorating the early pneumonitic phase. Either lower-dose or the higher-dose had the same effect.

RILI, which is also named as “radiation pneumonopathy” is a continuous process and regarded as the result of an abnormal healing response. Subclinical early damage in type I pneumocytes progress to an acute interstitial inflammation at 6-12 weeks after the onset of RT, which is called as “pneumonitic phase”. Radiation pneumonitis may be progressive and result

in chronic pulmonary damage or acute respiratory distress syndrome which eventually may be fatal. The radiation-induced acute lung parenchymal changes can progress to lung fibrosis after many months and years. Fibrosis is a part of the wound-healing process. Therefore, radiation fibrosis is a form of chronic lung damage that usually evolves over 4-24 months after irradiation [5,24]. Radiation-induced lung fibrosis is characterized by the accumulation of fibroblasts, myofibroblasts, inflammatory cells and extracellular matrix proteins, such as collagen, with the subsequent formation of a scar, which eventually results in impaired lung function [4, 25]. Because there are numerous contributing factors and various diagnostic settings for radiation-induced lung fibrosis, the reported incidence in patients who have received radiotherapy varies significantly, from 1% to 43% [26-28].

Imq, applied topically, has been used to treat morphea, a skin disease characterized by localized fibrosis, and its use has been advocated for the treatment of Dupuytren's contracture, another fibrosing disease [10, 21, 29-31]. Dytoc et al. reported their case series on the use of Imq for morphoea [21]. Morphoea or localized scleroderma is a fibrotic disease characterized by fibrosis of the skin with the accumulation of extracellular matrix (ECM) components, particularly collagen [21]. Research on fibrosis in patients with localized scleroderma focuses mainly on TGF-β, which is the master-chief of radiation-induced fibrosis. The results of the case series Dytoc et al. showed that Imq successfully treated the localized scleroderma. In the current study, Imq ameliorated the "fibrotic phase" of the RILI, without affecting the "pneumonitic phase". We found that TGF-β score of RT only group was significantly higher than low-dose Imq+RT; and high-dose Imq+RT groups scores.

Namazi postulated that Imq is a potential drug for Dupuytren's contracture, which is a condition of the hand characterized by the development of new fibrotic tissue in the form of nodules and cords [29]. In the pathophysiology of Dupuytren's contracture, TGF-β is a key fibrogenic cytokine that has been shown to stimulate fibroblast proliferation and extracellular matrix deposition [32]. By the same mechanism of radiation-induced lung fibrosis, Namazi hypothesized that Imq induces the cytokine IFN-α, IFN- γ, TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12 and it stimulate cell- mediated immunity (TH1 Pathway). IFN- γ is a TH-l cytokine that downregulates expression of TGF-β, which eventually ameliorates fibrosis process.

As mentioned before, "fibrosis" is part of the wound-healing process after an irritant stimulus in lung tissue. It was shown that adenosine receptors (AR) are required for appropriate granulation tissue formation and in adequate wound healing. A2A and A2B AR stimulate both of the critical functions in granulation tissue formation including new matrix production and angiogenesis; and the A1 AR may also contribute to new vessel formation [33]. Angiogenesis is a feature of chronic lung diseases such as asthma and pulmonary fibrosis. Studies in adenosine deaminase (ADA)-deficient mice, characterized by elevated lung tissue levels of adenosine, strongly suggest a causal association between adenosine and an inflammatory phenotype [33-35]. Schön et al. demonstrated that Imq, at pharmacologically relevant concentrations, is an A2AAR antagonist and that this may account for its immunological effects [31]. The end result of this additional activity of Imq would be an amelioration of fibrosis which may contribute to the anti-fibrotic effect of TGF-β-related pathway.

Our study has also some limitations that deserve to be mentioned as well. Firstly, we studied only the TGF-β related pathway without investigating the AR related pathway. We believe that, although the TGF-β related pathway seems to be the major one, AR-related pathway, which causes anti-fibrotic effect indirectly, worth to be investigated in a new study. Secondly, we did not demonstrate any difference with respect to pneumonitic phase between the RT groups. In the pathophysiology of the RILI, it was emphasized that the fibrosis is an abnormal wound-healing process, and usually seen as a progression of pneumonic phase. However, Imq itself causes pro-inflammatory activity which may be augmented by suppression of a negative feedback mechanism mediated by adenosine receptor signaling. Therefore, in order to explain better both limitations, there is a need for a new study investigating the radiation fibrosis and Imq induced anti-fibrotic activity at a molecular level.

Conclusion

The current study is the first one observing the effect of Imq on RILI. We demonstrated that Imq attenuated the radiation-induced lung fibrosis without ameliorating the radiation pneumonitis. Therefore, during the pathophysiology of RILI Imq have a limited anti-inflammatory effect and a substantial anti-fibrotic effect. We did not observe any difference between low-dose and high dose Imq; therefore, a lower dose may be adequate. The anti-fibrotic effects of Imq might have potentially been attributed to its inhibitory effects on adenosine receptors. These findings should be clarified with further preclinical and clinical studies.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

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